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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Enhancing Host Immunity Against Viral Infections

(72) Baral, Edward - Canada ;
Nagy, Eva - Canada ;
Dawood, Magdy - Canada ;
Berczi, Istvan - Canada ;

(71) UNIVERSITY OF MANITOBA - Canada ;
MANITOBA CANCER TREATMENT AND RESEARCH FOUNDATION - Canada ;

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ABSTRACT OF THE DISCLOSURE

Cell mediated immunity against persistent viral infection in a host is potentiated by administering to the host an effective amount of at least one antiestrogen capable of enhancing cytotoxic T lymphocytes in the host, such as a triphenylethylene tamoxifen, particularly tamoxifen or toremifene. This administration leads to the elimination of all infected cells and to the complete clearance of virus and virus induced tumor cells from the body, leaving protective cell mediated immunity. This therapy requires MHC restricted virus specific CTLs which then will kill virus infected very specifically, which may be initially present or formed *in vivo* or *in vitro*. The effect of the antiestrogen may be enhanced by the use of interferons optionally with indomethacin.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method for the potentiation of cell mediated immunity against persistent viral infection in a host, which comprises:

administering to a host an effective amount of at least one antiestrogen capable of enhancing cytotoxic T lymphocytes (CTLs) in the host.

2. The method of claim 1 wherein said host harbours MHC-restricted CTLs and said at least one antiestrogen is initially tested *in vitro* to demonstrate enhancement of lysis of virus infected cells.

3. The method of claim 2 wherein the antiestrogen is a triphenylethylene antiestrogen.

4. The method of claim 2 wherein said antiestrogen is tamoxifen or toremifene.

5. The method of claim 2 wherein said at least one antiestrogen is administered along with at least one interferon.

6. The method of claim 5 wherein said antiestrogen is tamoxifen or toremifene and said at least one interferon is interferon-α or interferon-γ.

7. The method of claim 6 wherein said at least one antiestrogen and at said at least one interferon are administered along with indomethacin.

8. The method of claim 1 wherein said host does not harbour MHC-restricted cytotoxic T lymphocytes, and MHC-restricted cytotoxic lymphocytes are activated and expanded in tissue culture and reinfused into the host and treatment with at least one antiestrogen is initiated with the first infusion of CTL and maintained for the entire treatment period.

9. The method of claim 8 wherein the antiestrogen is a triphenylethylene antiestrogen.

10. The method of claim 8 wherein said antiestrogen is tamoxifen or toremifene.

11. The method of claim 8 wherein said at least one antiestrogen is administered along with at least one interferon.

12. The method of claim 11 wherein said antiestrogen is tamoxifen or toremifene and said at least one interferon is interferon-α or interferon-γ.

13. The method of claim 12 wherein said at least one antiestrogen and at said at least one interferon are administered along with indomethacin.

14. The method of claim 1 wherein said host is initially treated with at least one immunostimulatory cytokine to stimulate MHC-restricted CTLs.

15. The method of claim 14 wherein said immunostimulatory cytokine is interleukin-2 or interleukin-12.

16. The method of claim 15 wherein the antiestrogen is a triphenylethylene antiestrogen.

17. The method of claim 15 wherein said antiestrogen is tamoxifen or toremifene.

18. The method of claim 15 wherein said at least one antiestrogen is administered along with at least one interferon.

19. The method of claim 18 wherein said antiestrogen is tamoxifen or toremifene and said at least one interferon is interferon-α or interferon-γ.

20. The method of claim 19 wherein said at least one antiestrogen and at said at least one interferon are administered along with indomethacin.

21. The method of claim 1 wherein said host is virus infected but otherwise immunocompetent, said host is vaccinated to stimulate MHC-restricted CTLs, and said host is tested for the presence of MHC-restricted CTLs prior to administration of said at least one antiestrogen.

22. The method of claim 21 wherein the antiestrogen is a triphenylethylene antiestrogen.

23. The method of claim 21 wherein said antiestrogen is tamoxifen or toremifene.

24. The method of claim 21 wherein said at least one antiestrogen is administered along with at least one interferon.

25. The method of claim 24 wherein said antiestrogen is tamoxifen or toremifene and said at least one interferon is interferon-α or interferon-γ.

26. The method of claim 25 wherein said at least one antiestrogen and at said at least one interferon are administered along with indomethacin.

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TITLE OF INVENTION

ENHANCING HOST IMMUNITY AGAINST VIRAL INFECTIONS

FIELD OF INVENTION

The present invention relates to immunology.

BACKGROUND OF INVENTION

Viruses carry infectious genetic material (either DNA or RNA) with the capacity of entering susceptible host cells and redirecting the biosynthetic machinery of the infected cells towards the multiplication of viral particles (virions). The profound conversion of the host cell toward viral production results in cell lysis in most cases (cytotoxic viruses), whereas in other cases the virus has no cytolytic effect, but rather persists within infected cells in the absence of morphological alteration. However, such virus infected cells may show a severe impairment of functions that are not necessary for cell survival (e.g. the secretion of hormones, antibodies, and other important molecules) (Fenner & White, 1976; de la Torre et al., 1991).

The DNA viruses most frequently causing persistent infections in man are *cytomegaloviruses*, the *Epstein-Barr virus*, *herpes viruses* (types I and II), *hepatitis viruses* (B, D), *papilloma viruses*, and occasionally *measles virus*. Recently, an association has been found between type II diabetes and the persistence of *cytomegalovirus* in the pancreas. *Herpes viruses* persist in nerve cells and may cause severe inflammatory disease in the nervous system and also in various other parts of the body after having travelled along the nerve fibers and triggering inflammation in the area of innervation of the fibers infected. The *Epstein-Barr virus* is known to cause infectious mononucleosis, and contributes to the pathogenesis of Burkitt's lymphoma, primarily in Africa, and nasopharyngeal carcinoma in the Orient. *Hepatitis viruses* cause inflammatory liver disease which may become

persistent in some individuals leading to cirrhosis. In addition, the hepatitis B virus has been identified as a contributing agent to the genesis of hepatocellular carcinomas. The measles virus may also persist in the nervous system and is known to cause the condition called *subacute sclerosing panencephalitis*. *Papilloma viruses* (Papova group) exist in many species, including man, and are known to cause common warts and genital tumors. Progressive multifocal encephalopathy is also caused by Papova virus (Fenner & White, 1976; Kimelman et al., 1985; Chow, 1993).

The common RNA viruses with the potential of causing persistent infections in man are *hepatitis A and C, rubella*, the *human T lymphocyte virus*, types I and II (HTLV), which are known to cause lymphomas, and the *human immunodeficiency virus* (HIV) which is recognized as the causative agent of acquired immunodeficiency syndrome (AIDS). HTLV and HIV possess an enzyme, called *reverse transcriptase*, which is capable of transcribing the viral RNA to a DNA template which in turn will permanently be inserted in the genome of the host cell. For this reason they are classified as *retroviruses*. Both DNA and RNA tumor viruses carry genes that are capable of growth stimulation of the host cell (viral oncogenes) (Fenner & White, 1976; Chow, 1993).

Persistent viral infections of farm animals that have economic significance are: *avian leukosis* caused by retroviruses (Okazaki et al., 1982); *bovine leukosis*, another retroviral disease (Daniel et al., 1993); *bovine viral diarrhea* (RNA pestivirus) (Thiel et al., 1993); *bluetongue*, a disease of ruminants transmitted by insects and caused by RNA viruses (arboviruses) (Sellers, 1981); *equine infectious anemia* caused by retroviruses (Sellon et al., 1994; Kennedy, 1992); retroviral disease of sheep caused by the *Maidi-Visna virus* (Kennedy, 1992); *porcine*

reproductive and respiratory syndrome caused by RNA lentivirus (Thiel et al., 1993).

Virus infected cells and virus induced tumors carry specific antigens on their surface which are recognized by the immune system of the host. These antigens may stimulate cell mediated and humoral immune reactions. Major histocompatibility complex antigen (MHC) restricted cytotoxic T lymphocytes, which are capable of specifically killing virus infected cells, are fundamental to host defence against viruses. However, it is recognized that natural killer cells and activated macrophages and the cytokines that are capable of activating these effector cells, namely IL-2, interferon-α and γ, and migration inhibitory factor, also contribute significantly to host defence against viral infections. Interferons α and γ have the capacity to inhibit the intracellular replication of viruses. Although some antibodies are capable of neutralizing viral particles, humoral immunity is considered to play a secondary role in host defence against viral infections (Fenner & White, 1976; Rinaldo & Torpey, 1993).

Initially, cell mediated immunity is present in immunocompetent hosts infected with persistent or oncogenic viruses. However, with time a gradual reduction occurs in the cell mediated antiviral/antitumor response, which is followed by nonprotective humoral responses, and some of the antibodies produced may actually interfere with cell mediated immunity (enhancing antibodies). Virus infected non-neoplastic cells may be similarly protected. In the case of tumors, immune derived growth factors may actually have a direct promoting effect on tumor growth which is known as immunostimulation. Apart from inducing a switch in the host immune response, which permits the persistence of viral infections and the growth of virus induced tumors, numerous other mechanisms have been proposed by which

immunodestruction of infected cells can be avoided. Some of these are: molecular mimicry, growing in immunologically privileged sites (such as the central nervous system), rendering immunocytes nonfunctional by direct infection, triggering nonspecific cytotoxic and autoimmune reactions (Oldstone & Rall, 1993; Tishon et al., 1993; Roger et al., 1994; Seligmann, 1990). HIV exposed individuals who are capable of resisting the establishment of persistent infection possess MHC-restricted cytotoxic T lymphocytes, but remain seronegative (Rowland-Jones et al., 1995).

Recent evidence indicates that virus specific CTL can be recovered from HIV infected individuals and from AIDS patients with active disease by *in vitro* stimulation with antigen and cytokines such as interleukin-2 or interleukin-12 (Clerici et al., 1993; Walker, 1989; Whiteside, 1993). Similarly, progressing neoplasms contain tumor infiltrating lymphocytes (TIL) which are inefficient in rejecting the tumor *in vivo*, but can be reactivated *in vitro* to exert tumor specific cytotoxicity (Berczi et al., 1973; Schendel & Gansbacher, 1993; Ioannides et al., 1993). Such reactivated CTL is being tested at present for the immunotherapy of cancer and of some viral infections (Kradin et al., 1989; Topalian et al., 1988; Torpey et al., 1993; Whiteside et al., 1993; Riddell et al., 1993).

SUMMARY OF THE INVENTION

We have discovered that the treatment of Epstein-Barr virus (EBV) transformed human B lymphocytes with the antiestrogenic agents, tamoxifen (TX) or toremifene (TO), enhances the destruction of these cells when used as targets for specific autologous CTL and LAK cells (see Tables 1 and 2 below). Currently, tamoxifen and toremifene are used in medicine for the treatment of estrogen receptor positive breast carcinomas and some

other estrogen dependent tumors. Human B lymphocytes do not express classical estrogen receptors, which suggests that the sensitizing effect is mediated by some other receptor.

Having regard to the new observations we have made, the present invention, in one aspect, provides a method for the potentiation of cell mediated immunity against persistent viral infections by administration to a host of at least one antiestrogenic agent capable of enhancing cytotoxic T lymphocytes (CTL), which is expected to lead to the elimination of all infected cells and to the complete clearance of the virus and virus induced tumor cells from the body, leaving behind protective cell mediated immunity. The prerequisite for this adjuvant therapy is the presence of MHC restricted virus specific CTL which kill virus infected target cells very specifically. The elimination of virus producing cells leads to virus clearance and lasting cell-mediated immunity. We showed earlier that TX is immunosuppressive (Nagy & Berczi, 1986; Baral et al., 1979). Therefore, TX and possibly TO, have a dual function during therapy with CTL, namely the sensitization of target cells and the inhibition of switch to humoral, nonprotective immune responses.

In accordance with one aspect of the invention, immunity in persistently infected hosts can be potentiated to cause viral clearance according to the following steps:

a. detecting MHC restricted cytotoxic T lymphocytes in the host,

b. demonstrating *in vitro* that antiestrogens enhance the killing of virus infected target cells, and

c. treating the infected host with an effective amount of at least one triphenylethylene antiestrogen to potentiate the killing of virus infected cells in a synergistic manner by CTL.

To fortify the immune response in hosts with advanced persistent viral infections in order to induce viral clearance and cure, the present invention, in another aspect, provides a method, as follows:

a. expanding killer cells in tissue culture and reinfusing to the host,

b. testing that antiestrogens enhance the cytotoxic action of these cells on virus infected targets, and

c. treating the host with an effective amount of at least one antiestrogen.

The enhancement of viral immunity in chronically infected hosts may be effected, in accordance with a further aspect of the invention as follows:

a. treating the host with an effective amount of at least one cytokine, such as interleukin-2 (IL-2) or interleukin-12 (IL-12), to induce and/or enhance cell mediated immune responses in the infected host,

b. demonstrating CTL activity and antiestrogen induced enhancement of virus infected target cell destruction, and

c. applying antiestrogen therapy in order to potentiate the killing effect of cytotoxic cells induced.

The treatment of hosts infected with persistent viruses may additionally be carried out as follows:

a. inducing virus specific CTL in the host by vaccination,

b. demonstrating CTL activity and antiestrogen induced enhancement of cytotoxicity, and

c. initiating antiestrogen therapy as soon as virus specific CTL is demonstrable in the immunized host.

Interferon-α (IFNα) is being used currently for the treatment of viral hepatitis and some other viral diseases. In order to take advantage of the anti-viral effect of interferon and the enhancement of host immunity by antiestrogens, we tested whether or not interferons

and antiestrogens can be applied jointly. As shown in Tables 3, 4 and 5 below, antiestrogens had a modest enhancing effect on the cytotoxic action of killer cells as did interferons. Joint application of interferons with anti-estrogens resulted in a further increase in cytotoxicity. Interferons are known to induce prostaglandins, which in turn can inhibit cytotoxicity. For this reason, we tested indomethacin (INDO) in order to see whether or not prostaglandin mediated inhibition exists in our system (Rossol et al., 1992). As shown in Tables 4 and 5 below, the joint application of antiestrogen plus interferon-γ plus indomethacin resulted in the highest cytotoxicity. This was true regardless of whether the killer cells were pretreated or when the drugs were applied during the cytotoxic reactions so that both target and effector cells were exposed to them. On the basis of these results, the following recommendations are made for treatment:

a. Use antiestrogen and interferon jointly to enhance host immunity and virus clearance in all situations listed above.

b. Additional treatment with indomethacin, especially in combination with interferon-γ and antiestrogen, is recommended for best results.

DETAILED DESCRIPTION OF THE INVENTION

A. General treatment protocols

1. Treatment of virus infected hosts in an early stage.

a. Virus infection is determined by the demonstration of viremia with the aid of tests for viral nucleic acid and/or viral antigen. An alternate method may be the detection of virus specific antibodies in the serum.

b. The presence in the infected host of major histocompatibility antigen restricted cytotoxic T

lymphocytes (MHCR-CTL) using *in vitro* methods of cytotoxicity is determined. If killer cell activity is found, the effect of TX and TO and other effective antiestrogens on the *in vitro* cytotoxic reaction is examined.

c. If MHCR-CTL is present in the host in sufficient level and TX and/or TO or other antiestrogen is found to sensitize virus infected target cells *in vitro* for immune cytolysis, the host is treated with at least one antiestrogen. Treatment with antiestrogen may be combined with treatment with at least one interferon and, optionally, indomethacin and maintained until virus clearance is achieved.

d. If no significant decrease in viral titer is observed in the blood during four weeks of treatment, additional treatment with interleukin-2 or interleukin-12, which are known to stimulate CTL activity, may be effected.

e. An alternate solution to the problem of insufficient viral clearance is the expansion of MHCR-CTL in tissue culture and reinfusion of the expanded CTL population to the host. Such treatment may be combined with cytokine treatment, such as with IL-2 and/or IL-12.

f. Vaccination may be an additional way to increase the level of MHCR-CTL in the host which could then be followed by treatment with antiestrogens in order to promote the destruction of infected cells and viral clearance.

2. Treatment of hosts with advanced infections but no apparent disease.

a. The diagnosis of infection and the presence of MHCR-CTL is done as described under 1. In the present case, it is expected that antibodies are present and nonspecific cytotoxic mechanisms also operate (for example, nonspecific $CD8^+$ CTL, antibody dependent cellular cytotoxicity, and complement fixing antibodies),

whereas MHCR-CTL may be weakly cytotoxic or completely paralyzed. Therapy is contraindicated with central nervous system involvement.

b. Reactivate MHCR-CTL *in vitro* using antigen presenting cells and cytokines, such as IL-2 and/or IL-12.

c. Establish that antiestrogens enhance cytotoxicity.

d. Expand the cultures and reinfuse into the host while antiestrogen treatment is applied.

e. Additional treatment with IL-2 and/or IL-12 in order to help maintain cytotoxicity at a significant level and treatment with interferons is recommended to limit virus multiplication.

3. Vaccination.

a. The difficulty of vaccination against persistent viral infections is that nonprotective or even enhancing immune reactions may also be induced, which would interfere with MHCR-CTL that mediate host response. Live virus vaccines that are capable of infecting the susceptible cells have been found to be far superior for vaccination than killed viruses. The apparent explanation for this is that only virus infected cells are able to present processed antigen associated with MHC-I which stimulate CTL specifically. Once a sufficient level of MHCR-CTL has been reached after the injection of the vaccine, and enhancement of cytotoxicity by antiestrogens established, antiestrogen treatment may be initiated in order to prevent the switch to humoral immune reactions. The vaccination of infected animals in combination with antiestrogen treatment is expected to lead to viral clearance and complete recovery which may not happen if the vaccine is given alone.

B. Methods.

We use HIV infection and AIDS as an example for our methodology. However, it will be apparent to those

skilled in the art that the methodology employed herein equally applies to other viral infections, including those described in the Background to the Invention.

1. **Diagnosis of infection.** Quantitative polymerase chain reaction for the detection of proviral DNA of HIV, a reliable assay for the detection of p24 viral antigen in plasma and assays for the detection of virus specific antibodies for routine tests are available (Wood et al., 1993). The DNA-PCR test is most useful for the follow-up of viral burden in patients.

2. **Detection of cytotoxic T lymphocytes.** MHCR-CTL can be assayed by using EBV-transformed B lymphocytes as targets that have been infected with a vaccinia virus carrying HIV genes. Both autologous and allogeneic B cells can readily be infected by such virus and by this means it is possible to test whether or not the CTL detected is MHC restricted (Lamhamedi-Cherradi et al., 1992; Grant et al., 1992; Ho et al., 1993). This test is also suitable for the determination of MHC restriction which is of utmost importance for our treatment protocol.

3. **Drug treatment.** For *in vitro* assays it is sufficient to treat target cells with TX (up to 1 μM) and TO (up to 5 μM) for 4 hours which is followed by labelling with ^{51}Cr and using the target cells in cytotoxicity assays. The most important virus carrying target in HIV infections is the $CD4^+$ helper T lymphocyte, although monocyte-macrophages, astrocytes, neurons and epithelial cells may also be infected. The enhancing effect of antiestrogens on the lysis of autologous HIV infected targets by MHCR-CTL is to be established prior to the initiation of treatment.

For the treatment of patients, the doses of TX and TO that are currently in use for cancer therapy may be applied. For TX, a suitable oral dose is about 20 to 40 mg given daily (Anderson et al., 1991). The therapeutic dose for TO is about 40 to 60 mg, but can be raised up to

about 240 mg daily (Hietanen et al., 1990). Antiestrogen treatment is to be started as soon as MHCR-CTL is present in the patient (for example, endogenous killer cells, vaccine induced killer cells or *in vitro* activated killer cells reinfused to the patient). The antiestrogen therapy and killer cell therapy (if given) are then maintained jointly, optimally until the complete clearance of the virus. Additional treatment with interferon would help to control virus production and enhance further host immunity.

4. **Treatment with cytokines.** Interleukin-2 has long been demonstrated to have a therapeutic effect on syngeneic animal tumors and some beneficial effects could also be demonstrated in certain human malignancies. Recently treatment of AIDS patients with IL-2 also produced beneficial effects, especially when used in combination with zidovudine (Schwartz et al., 1991; Mazza et al., 1992; McMahon et al., 1994). 0.2-12 x 10^6 IU/sq m doses were used and well tolerated when injected s.c.

Our survey of the cancer literature indicates that the dose of IL-2 which is well tolerated ranges from about 2 to 8 million IU (about 7.5 to 30,000 IU/kg) which is given subcutaneously (s.c) for about 4 to 5 days (often combined with killer cells). Fever, chills, skin rash, anxiety and dizziness may occur as complications of subcutaneous IL-2 treatment (Schoof et al., 1988; Mitchell et al., 1988; Topalian et al., 1988; Hancock, 1991; Sosman et al., 1988). We recommend treatment with 5 million IU/day s.c., which is maintained for 10 days while the patient is also treated with TX or TO or other antiestrogen. This treatment may be repeated several times in a week to 10 day intervals if it is deemed to be necessary while drug treatment is maintained continuously.

Interleukin-12 has been recognized recently as a cytokine with a powerful stimulatory effect of cell

mediated immunity, especially of CTL and NK cells. IL-12 has suppressed tumor growth in animal model systems and exerted antimicrobial activity against bacterial, yeast, parasitic and viral models of infection (Chehimi & Trinchieri, 1994; Wolf et al., 1994). IL-12 has also been shown to stimulate CTL responses to viral antigens such as influenza virus and HIV *in vitro*, and is capable of reactivating anergic CTL from HIV infected patients during *in vitro* stimulation (Clerici et al., 1993).

Currently, there is insufficient human experience with IL-12 treatment to recommend a schedule for dosage. It is known, however, that IL-12 is less toxic than is IL-2 so it is to be expected that the dosage would be more lenient. Once a suitable dose has been established, we would recommend to use an effective daily dosage for 10 day intervals jointly with TX or TO treatment similar to our recommendation for IL-2.

The treatment of chronic hepatitis C in patients with interferon-α is of current interest. The standard dose of IFNα 2b is 3 million units/week for 24 weeks. High doses (15 million units or 22.5-30 million units/week) for 24 weeks have also been tested. Higher doses of IFN ameliorated the severity of hepatitis in patients who did not respond to the standard dose; however, a sustained response was not produced as a rule. Side effects were increased with high doses (Bonkovsky et al., 1996). AIDS patients were treated with low dose IFNα (3 million units s.c., 3 x week). This dose was well tolerated and had a beneficial effect on HIV-related thrombocytopenic purpura (Northfelt et al., 1995). Interferon-γ has been used experimentally on cancer patients at doses of 150-200 μg s.c. (Lummen et al., 1996; Fiehn et al., 1995).

5. **Expansion of CTL in culture.** Peripheral blood lymphocytes are stimulated with autologous B lymphoblastoid cell lines derived by infection with the

Epstein-Barr virus followed by infection with recombinant vaccinia virus containing HIV genes. Such stimulator cells can be fixed with paraformaldehyde and used as antigen presenting cells to stimulate peripheral blood mononuclear cells from asymptomatic HIV seropositive individuals. Specific cytotoxic activity can be determined by ^{51}Cr release from viable antigen bearing B cell lines used for stimulation and from similar targets generated with B cell lines of unrelated subjects. Interleukin-2 is useful to aid expansion of CTL in this system. HIV specific CTL may be maintained for as long as 11 months and may be reinfused to the donor patients without any significant complications (Walker et al., 1989; Grant et al., 1992; Lamhamedi-Cherradi et al., 1992; Ahmad et al., 1993; Torpey et al., 1993; van Baalen et al., 1993; Whiteside et al., 1993).

6. **Adaptive immunotherapy of AIDS patients with *in vitro* expanded MHCR-CTL.** There is not enough experience with regard to the adaptive immunotherapy of AIDS patients with *in vitro* generated CTL for the establishment of a definite treatment schedule. However, in cancer immunotherapy there is somewhat more experience and a cell dose of about 1×10^8 to 1×10^{10} has been suggested as being effective for treatment with lymphokine activated killer cells. The cells may be infused every day or every other day for up to about 10 days. The patient may require about 1 to 4 such treatments sequentially before achieving tumor remission. CTL may be administered in a similar fashion (Rosenberg et al., 1987; Fujimoto, 1992; Horvath et al., 1993). We recommend daily intravenous infusion with about 5×10^9 to 10^{10} MHCR-CTL for 5 days, which is to be repeated in 10 day intervals, while the patient is maintained on TX or TO continuously. Additional treatment with interferon is desirable.

7. **Vaccination.** Hu et al. (1989) vaccinated macaques with recombinant vaccinia virus which expressed the envelope glycoprotein or simian type D retrovirus. The vaccinated animals showed complete protection when challenged with pathogenic doses of simian AIDS virus. HIV infected subjects have been vaccinated similarly with recombinant vaccinia virus expressing HIV glycoprotein 160. Such vaccination resulted in MHC restricted cytotoxic T lymphocytes showing specificity for gp160 (Kundu et al., 1992; el-Daher et al., 1993). In the anticipation that effective vaccine(s) will be developed against HIV in the future, we recommend the treatment schedule outlined below.

Once viral immunity (mediated by MHCR-CTL) has been induced in HIV infected patients, we recommend immediately the initiation of TX or TO treatment as described above. Additional treatment with IL-2 or IL-12 is also recommended in order to support the long term maintenance of virus specific MHC restricted CTL. If the level of CTL induced is judged to be unsatisfactory, one may consider the *in vitro* expansion of CTL and reinfusion as discussed above. Interferon treatment should be considered as an adjunct therapy, especially if the viral load is significant.

EXAMPLES

A series of experiments were carried out, the results of which are outlined in Tables 1 to 5 below.

Example 1:

This Example shows the effect of tamoxifen (TX) and toremifene (TO) on lysis of Epstein-Barr virus (EBV) by autologous cytotoxic T lymphocytes.

In a first set of experiments, cytotoxic lymphocytes were generated by co-culturing Epstein-Barr virus (EBV) transformed B lymphocytes with autologous peripheral blood mononuclear cells for 5 days or longer. Tamoxifen (TX) (1 μM) or toremifene (TO) (5 μM) treatment of the

target cells was effected for 4 hours, prior to labelling the target cells with ^{51}Cr. The cytotoxicity reaction was carried out at a 1:25 target:effector ratio for four hours.

The results of this first set of experiments is set forth in Table 1 below. As may be seen from this Table, TX and TO potentiated the lysis of EBV-transformed B lymphocytes by autologous cytotoxic T lymphocytes.

Example 2:

This Example shows the effects of TX and TO on lysis of Epstein-Barr virus (EBV) by autologous LAK cells.

In a second set of experiments, lymphokine activated killer (LAK) cells were generated by the stimulation of autologous peripheral blood lymphocytes (2×10^6/ml) with human recombinant interleukin-2 (500 IU/ml) for 6 days. TX (1 μM) and TO (5 μM) treatment of target cells was effected for 4 hours prior to labelling the cells with ^{51}Cr. The cytotoxicity reaction (1:25 effector:target ratio) was terminated at 4 hours.

The results of this second set of experiments are set forth in Table 2 below. As may be seen from Table 2, TX and TO potentiated the lysis of EBV-transformed B lymphocytes by autologous LAK cells.

Example 3:

This Example shows the effect of interferon on the cytotoxic action of TX and TO.

Next we tested whether or not treatment of the cytotoxic cells with TX, TO, or with IFNα would affect their cytotoxic action. The treatment shown in Table 3 was applied to CTL for 4 hours (TX at 1 μM, TO at 5 μM, and IFNα at 500 IU/ml), which was followed by cytotoxicity assay (^{51}Cr release) for another 4 hours at 1:25 target:effector ratios. As is shown in Table 3, interferon did increase the cytotoxic action of CTL as did TX and TO, and treatment with both agents resulted in the highest cytotoxicity.

Example 4:

This Example shows the effect of interferon-α, interferon-γ and indomethacin in combination with TO on target cells.

In the next series of experiments, we extended our studies for the treatment of target cells with interferon-α, interferon-γ (100 IU/ml), and indomethacin (5 μg/ml), in combination with TO (5 μM). The results are given in Table 4. Experiments with non-treated targets indicate that the treatment of effector cells with TO, IFN-α plus INDO raised significantly the mean cytotoxicity from 9.6 ± 1.5% to 22.5 ± 0.9% ($P<0.01$). When cytotoxicity on non-treated targets was compared to TO treated targets, significant enhancement occurred with CTL after TO treatment ($P<0.05$), after IFN-γ plus INDO treatment ($P<0.05$) and with CTL treated with TO plus IFN-γ plus INDO ($P<0.01$). These experiments indicate that the enhancement of cytotoxicity by TO will take place in the presence of interferon, and that interferon-γ especially in combination with indomethacin actually enhances significantly the cytotoxic action of killer cells (Table 4).

Example 5:

This Example shows the effect of IFN-α, IFN-γ, TO and INDO and combinations thereof.

Finally, we exposed both the target and the effector cells to IFN-α, IFN-γ, TO and INDO, and to their various combinations (at concentrations given for Table 5) for the duration of the cytotoxic interaction (6 or 18 hrs). In case of 6 hr treatment a significant elevation of cytotoxicity was observed with TO treatment alone, and TO plus INDO ($P<0.05$), IFN-α plus TO plus INDO and IFN-γ plus TO plus INDO ($P<0.01$). However, no further enhancement of the cytotoxic reaction was achieved by the addition of IFN and INDO compared to TO alone. The trend obtained in 18 hour cultures was similar. Here target cell destruction reached its highest value in the

cultures treated with TO plus IFN-γ plus INDO ($P<0.01$). These experiments indicate that both the antiestrogens (in this case, TO) can enhance the cytotoxic effect of CTL and that additional treatment with interferons and INDO does not interfere with this effect. Therefore, it seems feasible that one can enhance the destruction of virus infected target cells by antiestrogens while interfering with virus production using interferon-α or -γ. Taken together, these experiments indicate that the treatment of viral diseases, such as HIV infection or hepatitis C, with interferons can be improved if additional treatment with antiestrogens is applied. Indomethacin can be used to oppose the generation of immunosuppressive prostaglandin E by interferon treatment.

SUMMARY OF DISCLOSURE

In summary of this disclosure, a method is provided for the potentiation of cell-mediated immunity in an infected host against persistent viral infection by the administering to the host, which may be a human or animal host, an effective amount of at least one antiestrogenic agent, which may be a triphenylethylene antiestrogen, including tamoxifen and toremifene. This treatment is compatible with interferon therapy, which is known to limit the production of viral particles. Combination treatment with antiestrogens and interferon is expected to lead to better results than interferon treatment alone. Modifications are possible within the scope of this invention.

TABLE 1

The anti-estrogens, tamoxifen (TX) and toremifene (TO), enhance the killing of EBV infected target cells by autologous cytotoxic T lymphocytes (CTL).

Exp.	Treatment	Percent lysis	Isotope release (^{51}Cr cpm ± SE)	
			Spontaneous	Total
1	None	3±0.3	189±15	1925±141
	TX	22±1.2[c]	179±20	1889±94
	TO	18±0.6[b]	190±11	1912±73
52	None	14±1.1	177±12	1657±111
	TX	34±2[b]	184±25	1701±98
	TO	32±0.9[b]	196±17	1715±135
3	None	4±0	279±12	2703±48
	TX	15±0.6[b]	265±11	2561±71
	TO	14±0.3[b]	236±17	2447±94
4	None	3±0.3	289±20	2613±25
	TX	12±1.1[a]	274±16	2648±115
	TO	11±0.3[a]	280±15	2697±98
5	None	3±0	284±7	2645±145
	TX	14±1.1[b]	275±11	2579±94
	TO	12±0.3[a]	291±13	2701±111

Statistics (t test): Controls with no treatment were compared with groups treated with TX or TO.

a = P<0.05; b = P<0.01; c = P<0.001

TABLE 2

The antiestrogens, TX and TO, enhance the killing of EBV infected target cells by autologous lymphokine activated killer (LAK) cells.

Exp	Treatment	Percent lysis	Isotope release (^{51}Cr, cpm ± SE)	
			Spontaneous	Total
1	None	1±0	257±15	1678±114
	TX	10±0.6[b]	284±19	1934±198
	TO	16±1.5[c]	361±27	2067±157
2	None	3±0.3	238±31	2001±113
	TX	15±1.3[b]	274±25	2484±177
	TO	17±0.5[c]	297±18	2613±194

Statistics: Please see legends to Table 1.

TABLE 3

The effect of TX, TO and IFNα on the cytotoxic action of CTL.

Treatment of CTL	Percent lysis
none	1 ± 0
IFNα	7 ± 0.9[b]
TX	5 ± 0.7[a]
TO	8 ± 0.3[b]
IFNα + TX	18 ± 2.9[c]
IFNα + TO	29 ± 1.2[c]

Please see legends to Table 1.

TABLE 4

The effect of TO, IFNα, -γ and INDO on the cytotoxic potential of CTL.

Treatment of CTL Exp:	Percent lysis 1	2	Mean±SE[1]	3 (TO)	4 (TO)	Mean ±SE[2]
None	6±0.8	13±0.6	9.6±1.5	13±0.6	23 ± 1.1	18±2.3
IFNα	10±0.6	14±0.9	12±1.1	16±0.7	26 ± 0.9	21.5±2.3
IFNγ	11±0.9	16±0.3	13±5.1	18±1.1	27 ± 0.3	19.3±3.1
TO	17±0.9	14±0.3	16±0.8	24±0.6	29 ± 1.2	26.5±1.2
INDO	8±0.3	13±1.1	10.6±1.1	14±1.1	20 ± 0.6	17±1.4
IFNα + INDO	10±1.1	15±0.6	12.3±1.2	17±0.3	18 ± 1.1	17.3±0.6
IFNγ + INDO	13±0.9	20±0.6	16.6±1.5	24±0.9	30 ± 1.7	26.8±1.6
TO + INDO	17±1.1	16±0.3	16.6±0.6	27±1.5	31 ± 1.2	25.3±3.3
IFNα + INDO + TO	19±1.5	17±1.1	18±1	27±0.3	31 ± 1.7	24.1±4
IFNγ + INDO + TO	22±1.4	23±1.2	22.5±0.9[b]	32±1.1	39 ± 0.9	35.6±1.8[b]

[1] Mean±SE was calculated from exps 1 and 2, which were conducted with non-treated target cells.

[2] Mean±SE was calculated from exps 3 and 4, which were done with TO treated target cells.

Statistics (t test): Mean cytotoxicity of control groups compared to all other groups using the same target cells - untreated vs TO + IFNγ + INDO ($P<0.01$). Controls compared to all other groups - not significant. Mean cytotoxicity on untreated targets compared to TO treated targets - CTL treated with TO or IFNγ + INDO treatment ($P<0.05$); CTL treated with TO + IFNγ + INDO ($P<0.01$).

TABLE 5

The killing potential of CTL is increased by the presence during the cytotoxic reaction of TO, interferon-γ (IFNγ) and indomethacin (INDO).

Treatment	Percent lysis					
Exp:	1 (6 h)	2 (6 h)	Mean±SE[1]	3 (18 h)	4 (18 h)	Mean±SE[2]
None	9±0.7	10±1.1	9.3±0.6	17±1.5	20 ± 1.1	18.6±1
IFNα	7±0.3	10±0.3	8.8±0.7	16±0.9	18 ± 0.3	17±0.5
IFNγ	8±0.3	11±1.1	9.6±0.8	21±0.6	22 ± 0.6	21.5±0.4
TO	20±0.8	19±0.3	19.5±0.6[a]	25±2.9	29 ± 2.6	27±1.9
INDO	8±1.1	9±1.1	8.5±0.7	17±0.3	17 ± 1.1	17±0.5
IFNα + INDO	12±0.6	14±1.2	13±0.6	18±1.4	19 ± 1.2	18±0.9
IFNγ + INDO	14±0.3	19±0.6	16.3±1.2	18±1.2	23 ± 1.4	20.5±1.5
TO + INDO	18±0.9	20±0.6	18.6±0.9[a]	22±1.1	22 ± 1.1	22±0.7
IFNα + INDO + TO	20±1.1	20±1.1	20±1[b]	21±1.5	25 ± 0.9	23±1.2
IFNγ + INDO + TO	24±0.3	22±1.2	23±0.7[b]	29±1.4	33 ± 1.1	31±1.1[b]

[1] Mean cytotoxicity ± standard errors was calculated from experiments 1 and 2, which were conducted for 6 hours.

[2] Mean cytotoxicity ± standard errors was calculated from experiments 3 and 4, which were conducted for 18 hours.

Statistics (t test): Mean cytotoxicity of control groups with no treatment in the 6 hr experiment compared to all other groups - significant increase in cytotoxicity occurred with TO and TO + INDO ($P<0.05$), and IFNα + TO + INDO and IFNγ + TO + INDO ($P<0.01$). Controls compared to all other groups - not significant. In the 18 hr experiment, cytotoxicity is significantly higher than in controls in the IFNγ + TO + INDO treated group ($P<0.01$). The other groups are not significantly different.

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